Moll. Res. 18: 33–41 (1997).

Mineralisation in the teeth of the gastropod mollusc *Nerita atramentosa*

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Key words = biomineralisation, granules, radula teeth, silica, aluminium.

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Abstract

The radula teeth of the littoral gastropod, *Nerita atramentosa,* have been analysed using energy dispersive spectrometry. The matrix of the mature major lateral teeth contains sulfur, chlorine, potassium, calcium and magnesium. The teeth are tanned, and additionally hardened by the possession of biomineralised granules which contain sulfur, chlorine and calcium, together with a large range of other elements including silicon, aluminium, titanium, chromium, iron, nickel, and manganese. These granules, which are incorporated into the matrix of the teeth, result in a surface comparable to sandpaper which presumably serves to increase the abrasive properties of the major laterals. In contrast, the marginal teeth, which are only used for sweeping food particles into the mouth, are not mineralised to the same extent and do not contain granules. This approach to the biomineralisation of their teeth may well have contributed to the competitive success of *N. atrementosa* in the littoral environment.

Introduction

In Australia, as in many other parts of the world, the littoral zone is inhabited by a large variety of molluscs. In many cases these molluscs have specific adaptations which allow them to exploit more fully the environment in which they live. This is particularly the case with regard to feeding, where many species have hardened their teeth using a variety of materials. Such a process, known as biomineralisation, has classically been studied in the patellogastropod limpets and the chitons, though many other mollusc groups also use this technique. In the case of chitons, hardening is via the use of iron whereas both iron and silica are used in patellogastropods (Jones *et al.* 1935, Mann *et al.* 1986, Kim *et al.* 1989, Lowenstam & Weiner 1989, Webb *et al.* 1989). In both of these groups, the biomineralisation process results in teeth in which the organic matrix constitutes a relatively small proportion of the tooth biomass, with the majority of the tooth being infilled with the respective minerals.

Nerita atramentosa Reeve, 1855, is a gastropod mollusc common to the mid-littoral shore region, and whose distribution in Australia stretches from Queensland round the southern coast to the North-west Cape of Western Australia (Wells & Bryce 1986). *N. atramentosa* is a very successful competitor, able to grow faster and live longer than other mollusc species inhabiting the same niche

(Underwood 1978). The genus *Nerita* has a modified rhipidoglossan radula, in that the central rachidian and minor lateral teeth are reduced; the major lateral tooth has a single spoon-shaped cusp as opposed to the classical multi-cusped design; and there is a reduced number of marginal teeth (Hickman 1980). This modification of design presumably enables *Nerita* to scrape surface micro-algae from the rocks on which it lives (Hickman 1980). As such, the diet of *N. atramentosa* consists predominantly of diatoms and algal spores which are possibly replenished with each high tide (Underwood 1978). Though described as a surface film grazer (Underwood 1978), the excavational capabilities of its radula must be substantial, as its faeces are composed of 83% inorganic material (Black *et al.* 1988). In view of the role of biomineralisation in the teeth of chitons and limpets, both of which are known to possess exceptional excavating abilities (Steneck & Watling 1982), this study was initiated in order to determine the degree to which the teeth of *N. atramentosa* are mineralized with elements other than calcium. The possession of such biominerals in this species, which is taxonomically very distinct from the Patellogastropods and Polyplacophora, may well be a contributing factor to its competitive success.

Materials and Methods

Adult specimens of *Nerita atramentosa* (approximately 3 cm in overall shell diameter) were collected from intertidal rock platforms at Rottnest Island, Western Australia (latitude 32° S, longitude 116° E). Following dissection out of the animals, the radulae were cleaned with 5% w/v NaOCl for 10 minutes and fixed overnight using glutaraldehyde (3% by volume) in filtered seawater. For light microscopy samples were examined both prior to and following cleaning. All samples for scanning electron microscopy (SEM) were cleaned and fixed as above, dehydrated through a graded series of alcohols followed by amyl acetate, dried at the critical point of carbon dioxide, mounted on aluminium stubs and coated with either a thin layer of carbon for energy dispersive spectrometry (EDS) or with carbon and gold for photography. In addition, a number of individual lateral and marginal teeth were removed and mechanically damaged to expose a transverse cross section through the tooth core. These teeth were processed as for the whole radulae for SEM observation and EDS analysis. Light microscope measurements were made on a minimum of five animals, and analysis at the electron microscope level on a minimum of three animals. SEM was conducted using a Philips XL20 microscope, while EDS was undertaken using an EDAX 9100 system in the semi-quantitative mode, using both specific area as well as spot analysis for 100 seconds at 15 KeV. This system provides for the semi-quantitative analysis of all elements with an atomic weight greater than that of fluorine. As such, the results for each element are expressed as a relative proportion of all the elements selected for analysis. In order to ensure that a fully representative study was undertaken, specific area analysis was conducted on every fifth row of the radula, at five separate locations, on the major lateral teeth and the inner and outer marginal teeth. In addition, where marked colour changes occurred, every second row was similarly analysed. Spot analyses were conducted when backscattered images indicated the presence of higher atomic weight elements than those normally occurring in the surrounding matrix.

Preliminary analysis revealed the presence of silicon, and in order to further characterise the mineralisation involved, radulae from four animals were dissected out and washed in 0.05M NaOCl for 30 minutes. In order to digest the organic matrix and acid-soluble mineralisation products, the radulae were then incubated in concentrated HCl at 50 °C for 40 minutes, and the remains collected by centrifugation at 16000 g for five minutes (IEC Minimax). The pellet was washed a further five times by resuspension in distilled water, reconcentrated by centrifugation at 16000 g for two minutes, frozen at -80 °C for 10 minutes and then freeze dried overnight. The white fibrous solid that remained after this process was then transferred to a SEM stub coated with carbon impregnated double-sided sticky tape, and viewed under the microscope using both secondary and backscatter detectors. To prevent contamination, all procedures were conducted using plastic utensils and containers.

Figure 1. Scanning electron micrograph (SEM) taken towards the mature end of the radula of *Nerita atramentosa,* showing the enlarged major lateral teeth and the comb-like marginals. Note the major laterals show the presence of a disrupted cuticular layer. Scale bar = 500 μm.

Results

When removed from the animal, the radula of an adult *N. atramentosa* is approximately 26 mm long, 1.6 mm wide and consists of an average of 193 rows of teeth. Each row comprises a small central tooth flanked by a series of relatively flat lateral teeth, followed by the major lateral tooth which has a prominent spade shaped cusp (Fig. 1). On the outer edges of each row are the marginals which consist of a series of fine comb-like teeth (Fig. 1). The radula undergoes progressive maturation down its length, culminating in the teeth that are actually in use. In a freshly dissected radula, the immature major lateral teeth are soft, colourless and transparent in appearance. By approximately row 120, these teeth are yellow in colour, changing progressively to green/black over the next seven rows. From this stage onwards, the teeth are rigid enough to maintain a uniform shape in air. The tips of the inner two thirds of the marginal comb-like teeth exhibit the same colouration as the major laterals. Worn or broken teeth are appreciably lighter in colour. Cleaning in NaOCl results in all coloured teeth changing to a uniform brown. The final ten rows of the radula show progressively increasing patterns of wear towards the mature end, with the last four rows possessing marginal teeth only, the other teeth presumably being lost through wear.

High power examination under the SEM confirmed the picture seen at low magnification, revealing that the major lateral teeth consist of a single cusp with a prominent cutting edge (Fig. 2). In addition, the posterior surface of the non-working mature major lateral teeth exhibit a large number of fine serrations along the cutting edge (Fig. 2), although these are lost as soon as the tooth is used and thus play no functional role in feeding (Hickman 1980). The six most mature teeth show extreme wear. The underlying matrix of the major lateral teeth appears to be covered with a cuticle or surface layer which was presumably disrupted during processing (Fig. 1). A large number of granules, varying in diameter from 0.3 – 1.5 μm, were observed both on the anterior surface close to the cutting edge, and across the whole of the posterior surface of the tooth. These were embedded in

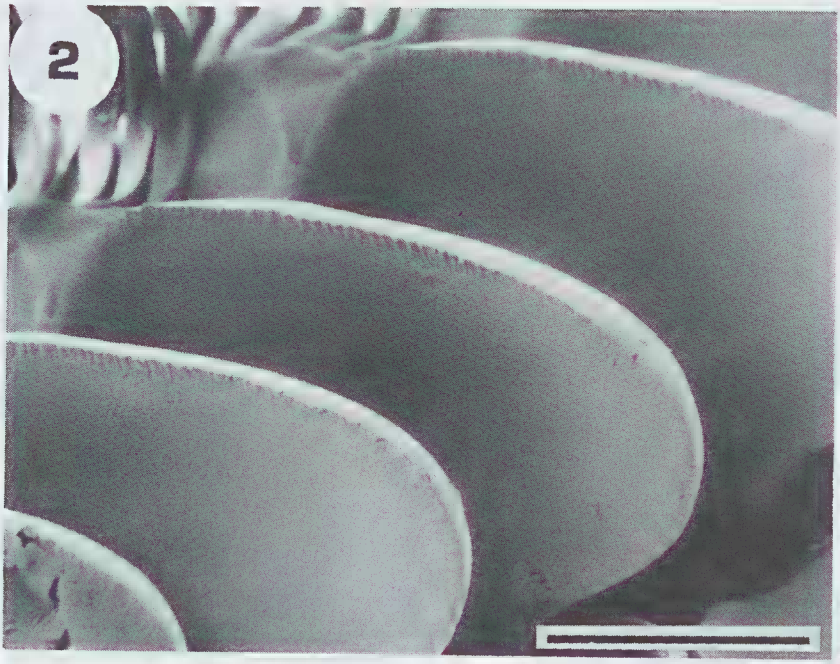

Figure 2. SEM of unused mature major lateral teeth showing the presence of serrations on the tip. Small granules can seen on the posterior surface of the teeth. Scale bar = 100 µm.

the tooth matrix, and also associated with the cuticle (Fig. 3). Use of the electron beam to etch away the outer layers of the posterior surface of unused teeth revealed that the granules were not just located on the surface of the major lateral teeth, but were embedded deep within the matrix. When the backscatter detector was utilised, very bright areas were observed, corresponding to the position of these granules, and indicating that they are composed of material containing elements of higher atomic weight than those in the surrounding matrix (Fig. 4).

Specific area EDS analysis of the posterior tips of the major lateral teeth at the immature end of the radula, revealed that chlorine and potassium were the only analysable elements present (Table 1). Sulfur was first apparent by row 120, and the ratio of sulfur to chlorine reversed with progression towards the mature end of the radula. Hence, with the exception of row 183 in which the teeth were clearly worn, from row 150 on, sulfur was the dominant element present. The mature teeth also contained large amounts of calcium and magnesium, together with aluminium and silicon and small amounts of phosphorous, potassium and titanium (Table 1). Spot EDS analysis of the mature major

Table 1. Specific area analysis of the tips of the posterior surface of the major lateral teeth. The composition of each element is expressed as a weight percent of all elements analysed.

	Mg	Al	Si	P	S	Cl	K	Ca	Ti
Row 100	0	0	0	0	0	66.0	34.0	0	0
Row 120	0	0	0	0	2.7	85.7	11.6	0	0
Row 130	16.9	10.3	9.6	0	18.2	28.0	1.9	14.9	0.2
Row 150	14.4	7.3	6.3	3.2	28.7	21.8	1.7	16.1	0.5
Row 170	12.2	7.3	6.3	3.0	31.9	20.2	1.4	17.6	0.1
Row 183	12.0	8.6	7.9	4.2	28.9	22.2	2.1	13.9	0.2

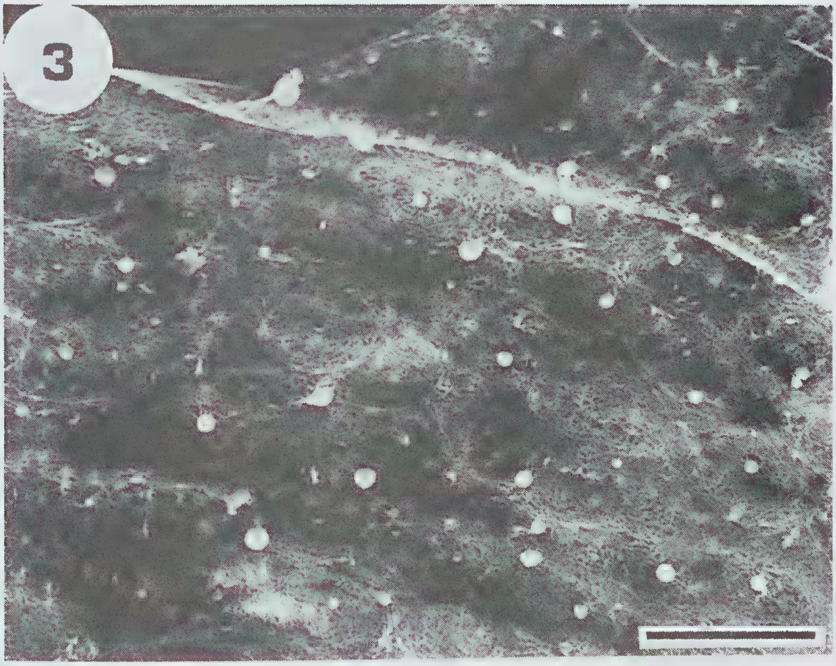

Figure 3. High power, secondary electron image of the posterior surface of an unused, major lateral tooth showing the presence of granules both on the surface, and embedded in the matrix. Scale bar = 10 μm.

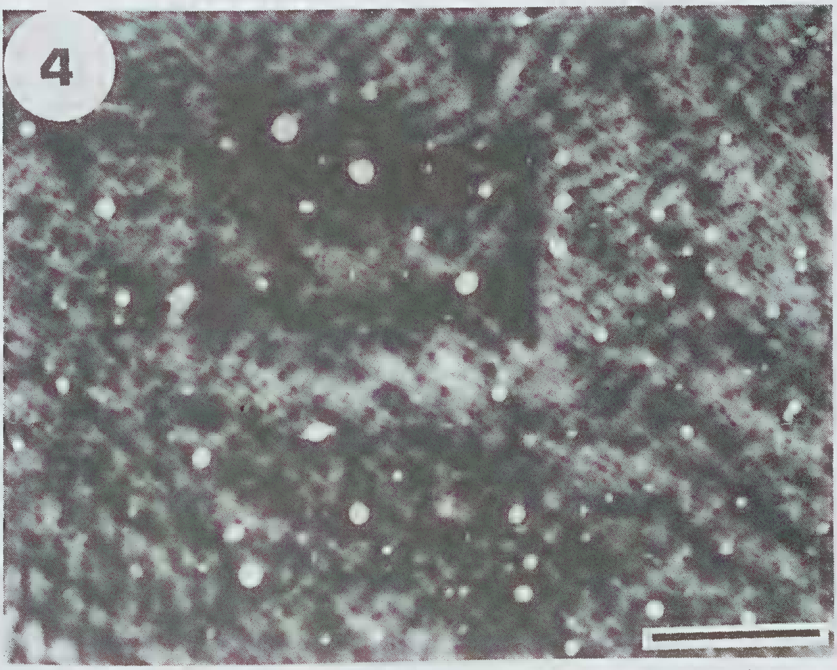

Figure 4. High power, backscattered electron image of the same region as in Figure 3, showing bright areas corresponding to the position of the granules, and which are indicative of the presence of higher atomic weight elements. Scale bar = 10 μm.

Table 2. Specific area analysis of inner and outer marginal (comb) teeth at row 170. The composition of each element is expressed as a weight percent of all elements analysed.

	Mg	Al	Si	P	S	Cl	K	Ca	Ti
Inner	10.3	4.9	3.7	1.6	22.6	39.2	2.4	15.2	0.1
Outer	8.8	4.8	3.0	4.8	11.2	42.6	4.6	20.2	0.2

lateral teeth, conducted away from the granules, revealed only the presence of sulfur, chlorine, potassium, calcium and magnesium. However, when the granules themselves were similarly analysed, a different picture emerged. Thus, while neither magnesium nor potassium were detected, in addition to sulfur, chlorine and calcium, a large range of other elements was found including silicon, aluminium, titanium, chromium, iron, nickel, and manganese, with the exact composition depending on the specific granule analysed.

The marginal teeth resemble a comb in structure consisting of approximately 35 individual teeth. The inner 25 possess smooth tips, while the outer 10 exhibit a serrated margin. Additional marginal teeth at the edge of the radula are reduced in size and fused together. Specific area analysis of all immature marginal teeth revealed that they contained chlorine and potassium, with calcium first being detected by row 100. The mature inner marginal teeth, corresponding to the coloured marginals seen under the light microscope, contained chlorine, sulfur, calcium and magnesium, together with aluminium and silicon, and small amounts of phosphorous, potassium and titanium (Table 2). The colourless, mature, outer marginal teeth contained the same elements with the major difference in composition being that they possessed considerably less sulfur (Table 2). No apparent difference in composition was found between different rows of mature marginal teeth down the length of the radula.

When mechanically fractured major lateral teeth were viewed using both the secondary and backscattered detectors, no compositional differences were detected between their inner and outer regions. Similarly fractured marginal teeth appeared relatively homogeneous throughout, with no granules observed in either intact or broken marginal teeth and no bright spots seen using the backscattered detector.

Acid digestion of the teeth resulted in a white particulate material with a fibrous appearance. Examination using SEM revealed that it was composed of a large number of highly reflective particles with a size range of 0.2 – 1.0 µm, similar to that of the granules embedded in the mature major lateral teeth. EDS analysis of these particles revealed that a large number contained appreciable amounts of silicon, aluminium and titanium. However, as occurred when individual granules were analysed, a large variety of other elements was detected, with their relative proportions depending on the particle selected for analysis.

Discussion

The results detailed in this study have shown for the first time that the major lateral teeth of *Nerita atramentosa* incorporate small calcified granules which differ, both in structure and elemental composition, from the overall tooth matrix. The distribution of these granules, restricted as they are to the matrix of the anterior tip and posterior side of the major laterals, which are the main scraping teeth, suggests that they are involved in increasing the abrasive properties of the tooth as a whole. Indeed, as has been suggested for patellogastropod limpets (Vincent 1980), our studies have shown that once the teeth are in use, the matrix is preferentially worn away to expose the granules, giving a surface comparable to sandpaper. The presence of considerable quantities of magnesium in the tooth matrix, compared to its total absence in the granules, implies that its exclusion imparts very different structural properties to these granules. A similar situation has been described for the Perciform fishes where magnesium, present in the bulk of the tooth, is conspicuously absent from the cutting edge, which is described as being much harder (Suga *et al.* 1992). The presence, following acid-digestion, of a residue composed of particulates, similar in size to the tooth granules, suggests that some of the minerals which occur in the granules differ in their chemical nature from those which are found in the matrix of the tooth. In the teeth of limpets, it has been suggested that silicon may be bound within the organic matrix prior to the impregnation of amorphous silica (Mann *et al.* 1986). The continued presence of silicon and aluminium in the acid-digested particles is indicative that these elements may be present in the granules in the form of amorphous silica and/or aluminosilicates. Although the benefit of the sandpaper approach to biomineralisation in individual teeth may be short-lived, this is compensated for by the radula in many molluscs, including *Nerita*, having a relatively rapid rate of tooth turnover (Runham 1962, Fretter 1965).

In *N. atramentosa,* the radula is composed of variously shaped teeth with specialised roles and mechanical properties. As in other neritids, the major lateral teeth are considerably enlarged and serve as the main scraping implements (Baker 1923, Fretter 1965), with the working teeth exhibiting extreme wear and being lost prior to adjacent teeth of the same row. Throughout the radula, the increasing colour observed at the light microscope level corresponds to a relative increase in the amount of sulfur. The presence of sulfhydryl groups has been associated with tanning, both in the radulas of other molluscs, and in arthropod cuticle (Runham 1963, Wigglesworth 1972). Hence, it is assumed that the presence of colour is indicative of cross-linking of the constituents of the radula matrix. Additional hardening of these teeth is achieved by the incorporation of biomineralised granules. The less robust marginal teeth exhibit little wear, and are only responsible for sweeping food into the mouth as the radula is retracted (Fretter 1965, Steneck & Watling 1982). The inner mature marginals are coloured (and presumably tanned), and mineralised to a level comparable to that which occurs relatively early in the development of the major lateral teeth. The serrated outer marginal teeth only contain half the amount of sulfur found in the inner marginals, and are not mineralised to the same extent, presumably reflecting their roles in particle size selection of food and as accessory sweepers (Hickman 1980). Despite the marginal teeth possessing the same range of elements as the major laterals, no granules were observed in either intact or broken marginal teeth and no bright spots could be seen using the backscatter detector. This observation lends further support to the suggestion that there is a link between the presence of the granules themselves, and the abrasive function of the major lateral teeth.

The presence of granules of a similar size to those recorded in this study, and also containing a variety of elements including heavy metals, is well documented in invertebrates, where they have a role in detoxification processes (see *e.g.* Taylor & Simkiss 1984). Indeed, this may have been their original purpose in *N. atramentosa*, as they would rapidly be removed from the body through radula tooth turnover (Runham 1962). The need for such a mechanism may have arisen as a result of the diatom rich diet of *N. atramentosa* (Underwood 1978). Diatom frustules are primarily composed of silica, with other elements such as aluminium, iron and titanium naturally bound to the surface (Werner 1977). Hence the ingestion of diatoms could account for the presence of these elements in the tooth granules. Aluminium and silicon have also been reported as occurring in the rectal gland granules of the sea urchin *Echinocardium* (Brown 1982). However, if the granules in *N. atramentosa* originally played a primary excretory role, some modification in their composition must have occurred, as magnesium, which is a relatively common component of excretory granules, is present in the tooth matrix, yet is absent from the granules.

The incorporation of hardening minerals into the teeth of animals has been known to take a variety of forms. For example, in chitons and limpets the mineral products occupy a discrete architectural compartment within the overall tooth structure (Mann *et al.* 1986, Kim *et al.* 1989, Lowenstam & Weiner 1989). In rats and tetra-odontiform fishes, the minerals are incorporated as part of the matrix structure (Selvig & Halse 1975, Suga *et al.* 1989), while in spiders and chaetodontid fishes they form a cuticular layer covering the core (Schofield & Lefrevre 1989, Sparks *et al.* 1990). All three approaches enable the exploitation of a resource that would otherwise prove difficult, or indeed impossible to use. In Western Australia, *N. atramentosa* occupies the same niche as the chiton *A. hirtosa,* with which comparisons can be made. Both species possess a similar radula design, in that the major lateral teeth are enlarged and have a reduced number of contact points with the substratum. In addition, both species have employed biomineralisation over and above the simple deposition of calcium carbonate, to enable them to browse the hard substrate on which they subsist. The excavating abilities of their respective radulae is demonstrated by the faeces of both species containing between 80 and 90% inorganic material (Black *et al.* 1988, Macey *et al.* 1996), though a proportion of this may result from the acquisition of material previously loosened by filamentous and boring algae (Ginsburg 1953, Lowenstam 1962, Raffaelli 1985). The success of *N. atramentosa* has been attributed to its fast rate of movement and rapid feeding (Underwood 1978). While the latter could be partially attributed to the size of the radula teeth (Black *et al.* 1988), the increased tooth hardness, through the possession of mineralised granules, may also play a significant role.

Acknowledgements

The authors would like to thank Dr. Winston Ponder of The Australian Museum, Sydney, for providing the inspiration and original samples for this study, and Assoc. Prof. John Webb of Murdoch University, Perth, for critical comments on the manuscript.

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